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Abstract

Polyhydroxyalkanoates (PHA) are bacterial polyesters usually produced from costly sugars or volatile fatty acids (VFAs). In this work, two processing waters rich in vegetable proteins and reducing sugars, *i.e.* a mixture of saccharose and stachyose in Leguminous Processing Water (LPW) and a mixture of glucose and fructose in Fruit Processing Water (FPW), were tested as growth medium for PHA production in a two-stage fermentation with a unique marine bacterial species: *Halomonas* i4786. In preliminary shake flask experiments, it was shown that the two media can effectively support the bacterial growth and the accumulation of PHA (evaluated using Nile Red staining). In batch cultivation mode in a 5-L fermentor, PHA productivities of 1.6 $g.L^{-1}$ and 1.8 $g.L^{-1}$ were further achieved within 72h, in LPW and FPW respectively. Polymer characterization by Differential Scanning Calorimetry and Steric Exclusion Chromatography indicated that the two substrates led to the biosynthesis of polymers with different chain length, distribution and crystallinity. To summarize, these results show that by-products derived from local agri-food industry can be used as a user-adapted and cost-effective source to produce bio-sourced and biodegradable plastic materials.

Keywords: Agro-industrial processing waters, Polyhydroxyalkanoates, Marine bacterium, Batch fermentation

Abbreviations: PHA, polyhydroxyalkanoates; LPW, Leguminous Processing Water; FPW, Fruit

Processing Water; NR, Nile Red; VFA, volatile fatty acids; DSC, Differential Scanning Calorimetry; SEC, Steric Exclusion Chromatography; FI, Fluorescence intensity; CDW, Cell Dry Weight.

- **Introduction**

In the general context of sustainable development, the formulation of bio-based and biodegradable plastics has given rise to increasing interest. Throughout the world today, the development of bio-based and biodegradable materials with controlled properties has been a subject of scientific and industrial research. These materials tend to substitute synthetic plastics in many applications that cause huge amount of waste, like as for example packaging. Among these bio-sourced and biodegradable polymers, the family of polyhydroxyalkanoates (PHA) is one of the most studied. PHA are microbial polyesters produced by numerous bacteria in nature as intracellular reserve of carbon or energy [1]. They are recognized as completely biosynthetic and biodegradable with zero toxic waste and recyclable into organic waste. PHA are efficiently degraded in the environment because many micro-organisms in the soil are able to secrete PHA depolymerase enzymes that hydrolyze the polymer ester bonds. Micro-organisms then metabolize these degradation products into water and carbon dioxide [2,3]. Moreover, they are also well known for presenting good biocompatibility, making them attractive as biomaterials.

Until to now, the major challenge is to reduce the cost of the biosynthesis process to make PHA bio-plastics economically competitive with oil-derived plastics. For this purpose, the use of inexpensive and readily available sub-products or wastes as bacterial growth substrate feedstock is focusing scientific and industrial interest regarding the subsequent deduction of the overall costs of production [4]. Besides, a feed of waste resource is beneficial from a life-cycle perspective. During the last decade, various waste streams from the food or agro-industry have been found to be possible substrates in the production of PHA due to their composition of more or less readily degradable organic molecules. Thus, a whole range of non-edible and waste cooking oils were successfully converted into PHA by *Cupriavidus necator* or various strains of *Pseudomonas* [5-8]. Usually, the bio-produced PHA have a relative wide range of C4 to C16 monomers. The potential of PHA production from dairy products, especially whey and whey hydrolysates, using common PHA-producing bacteria, has also been exploited in many works [9,10]. Molasses, oils or lignocellulosic raw materials have been described as others possible carbon-sources for the fermentative production of bioplastics [11-16]. In many cases, pre-treatment steps of the waste, *i.e.* filtration, saponification or enzymatic hydrolysis, are required for converting the residual polysaccharides or lipids into fermentable nutrients. Table 1 summarizes some combinations between different carbon sources and micro-organisms leading to PHA produced by pure culture from

waste raw materials.

PHA production from agri-food processing waters provides another alternative approach that has been yet scarcely investigated. In this study, two types of processing waters from leguminous (LPW) and fruits (FPW) industries were identified as high potential waste streams based on the criteria of abundance, local availability and high content of reducing sugars.

This paper describes: (i) the growth kinetics of a unique marine bacterium using LPW and FPW as growth media for batch fermentation, (ii) the detection of PHA inside the biomass using the Nile Red staining, (iii) the PHA production at the bioreactor scale, (iv) the physico-chemical characteristics of the produced biopolymers.

- **Materials and methods**
  - *Chemicals*

All the culture media components were purchased from Difco Laboratories (Detroit, MI) except the saltwater (Instant Ocean, United Pet Group, Cincinnati, OH). Other chemicals used in this study were analytical grade and obtained either from Sigma Chemical Co. (St. Louis, MO) or Thermo Fisher Scientific Inc. (Waltham, MA).

  - *Bacterial strain*

A moderately halophilic bacterial species belonging to the genus *Halomonas* and named *H.* i4786 was used to evaluate the feasibility of PHA production using agro-industry effluents. This bacterium has been isolated from Brittany (France) coastal sea water and has been characterized as a gram negative and non-spore forming PHA-producer under nitrogen limitation. The cells were grown in marine broth and stored as glycerol stocks (20 % v/v) at -80°C until being used.

  - *Agro-industrial by-products*

The nutritional composition of the leguminous (LPW) and fruit (FPW) processing waters tested as growth media for PHA production are displayed below (Table 2).

  - *Cultivation conditions for PHA production in flasks*

One vial of stock culture was used to inoculate a 250-mL Erlenmeyer flask containing 100mL of growing medium containing dehydrated LPW or FWP (20 g/L) as the sole carbon source in addition with tryptone (8 g/L), yeast extract (2 g/L) and saltwater (11 g/L). Marine broth (glucose 10 g/L, tryptone 1 g/L, yeast extract 0.5 g/L, saltwater 11 g/L), pH 7.5, was used as reference medium. After 17h at 25°C with orbital shaking at 200 rpm, the cultivation broth was centrifuged (7500 rpm,

10 min). The bacterial pellet was washed twice with PBS and then suspended in 100 mL of production medium (glucose 20 g/L, tryptone 20 g/L, yeast extract 0.4 g/L, saltwater 11 g/L). The fermentation was carried-out for 72 h during whom the broth was regularly sampled for analysis.

- *Cultivation conditions for PHA production in a fermentor*

The PHA production was performed in three separate stages: pre-culture, biomass production and PHA accumulation. First, the bacterial cells were grown in a 2L Erlenmeyer flask containing 200 mL of marine broth medium. After 7h at 25°C and a 200 rpm orbital shaking, the pre-culture was transferred to a 5L bioreactor (Biostat B plus, Sartorius, Germany) containing a final volume of 2L of LPW or FPW growing medium. During the process, the temperature was kept at 25 ± 1°C, antifoam (a single drop) was added and the pH was maintained at 7.5 ± 0.1 using 1M HCl and 1M NaOH. The agitation speed was set to 400rpm. Dissolved oxygen concentration was maintained above 50% of air saturation pressure by adjusting the aeration rate. After 17h, the cultivation was stopped and centrifuged at 7500 rpm at 4°C for 10 min and washed twice with PBS. The third stage was performed by inoculating 2L of PHA production medium by the bacterial cell pellet. The incubation conditions were identical as that of the previous stage except that the agitation speed was slowed down to 200 rpm.

- *Quantitative analysis*

PHA biosynthesis was evaluated using the Nile Red (NR) staining procedure adapted from Spiekermann *et al*. [17]. NR, from a stock solution at 0.25 $mg.mL^{-1}$ in DMSO, was added to the accumulation medium at final concentration of 1 $mg.L^{-1}$. Fluorescence emitted from the stained cells was recorded. 2 mL of the fermentation broth were collected, washed twice with PBS and diluted to an optical density at 543 nm ($OD_{543nm,\ l=0.4cm}$) of 0.15. Fluorescence emission spectra were collected by using a SLM 8100 spectrofluorometer, at 20°C between 560 and 720 nm at the fixed excitation wavelength of 543 nm. Spectra were then corrected by subtracting the contribution of the buffer to the recorded spectra and normalized with the OD_{543} value.

The number of viable cells ($CFU.mL^{-1}$) was determined by spread plating on marine agar plates of 100 µL of ten-fold serial dilutions of the culture broth.

Cell dry weight (CDW) was determined gravimetrically after centrifugation, washing and dry-freezing of the cell pellet. CDW was expressed as $g.L^{-1}$ of culture medium.

- *PHA extraction and characterization*

PHA extraction and purification were performed as described by Chardron *et al.* (2010) [18]. PHA was isolated from the lyophilized cells in an excess of dichloromethane (60 mL of solvent per g of

dry biomass) at 40°C for 24h. The polymer solution was filtered through a 0.45 µm cellulose nitrate membrane to remove cell debris and concentrated by rotary evaporation. PHA were precipitated twice into cold methanol (1:10, v/v) and dried in an oven at 50°C until a constant weight. The PHA content (% w/w) is defined as the PHA to CDW percent ratio.

PHA molar mass distribution was determined by Size Exclusion Chromatography (SEC). The stationary phase was composed of three column from Polymer Labs: 2 x ResiPore and 1 x PL gel Mixed C. The chloroform was used as eluent at a temperature of 45°C and a flow rate of 0.8 $ml.min^{-1}$. Detection system was composed of refractometer and a UV detector. The elution profiles were analysed by the software Empower GPC module (Waters). Molecular mass calculations were based on calibration curves obtained from polystyrene standards with molecular weights ranging from 580 $g.mol^{-1}$ up to 900 000 $g.mol^{-1}$. The weight-average molecular weight () and number-average molecular weight () were determined using the software Empower GPC module (Waters). The polydispersity index (*Đ*) is calculated as /.

Differential Scanning Calorimetry (DSC) analyses were performed by using a Mettler-Toledo DSC-882 equipment to determine PHAs thermal properties such as glass transition (T_g), melting temperature (T_m), crystallization temperature (T_c), melting enthalpy (ΔH_m) and crystalline ratio (X_c), as previously described [19].

The composition of the polyesters produced was determined by gaz chromatography (GC). The precipitate PHA was first subjected to methanolysis. Approximately 8 mg of precipitate was methyl esterified (4 h at 100 °C) with a solution consisting of 1.7 ml of methanol, 0.3 ml of 98% sulphuric acid and 2 ml of chloroform. After phase separation, the organic phase (bottom) was washed with water (1 mL) and dried with anhydrous sodium sulphate. Samples were injected on a Perkin Elmer Clarus 480 gas chromatograph equipped with a 30 m x 0.32 mm DB-5 (0.25µm film) column (HP) with splitless injector and flame ionization detector (FID). The oven temperature was kept at 70°C for 3 min followed by a ramp of 10°C/min to 240°C. It was held at this temperature for 10 min. The nitrogen flow was 0.9 mL/min at 70°C.

- **Results**

  - *Bacterial growth*

For an efficient and maximized PHA biosynthesis, the growing medium must be optimized as it is known to affect both the specific growth rates (μ) and the biomass yields. Accordingly, the LPW and FPW growing media were tested for their potential to support the bacterial growth in the PHA production process by *Halomonas* i4786 in comparison with the conventional marine broth. As displayed in Fig. 1, the stationary phase was reached within 10 hours of culture whatever the

growing medium. The total biomass obtained in the three cultures was only slightly different: 1.4 10^8 and 7.2 10^8 CFU.mL^{-1} for FPW and LPW growth media respectively as compared to 3.0 10^8 CFU.mL^{-1} in marine broth containing 10 g.L^{-1} glucose. Such result assesses the capacity of the bacteria to metabolize the reducing sugars that are contained in the processing waters. Besides, the specific growth rates of 0.82 h^{-1} in FPW and 2.22 h^{-1} in LPW indicate that these media allowed the culture to develop about 1.3 to 3.6 times more rapidly than in marine broth (μ = 0.61 h^{-1}).

- *PHA accumulation in shake flasks*

Previously, *Halomonas* i4786 was identified as a P(3-HB) bacterial producer under nitrogen limitation [20]. In order to assess if *Halomonas* i4786 was able to accumulate PHA after a growth phase on agro-industrial substrates, the Nile Red staining procedure was employed as described in section 2.6. As shown in Fig. 2, the three fluorescence emission spectra, recorded after 72h of cultivation, exhibit a same emission band centred at 592 nm. However the maximal fluorescence intensity (FI) was lower with the culture that was first grown on marine broth (FI = 0.56) than those grown on LPW (FI = 0.85) and FLW (FI = 1.26). Besides, PHA production seems to be more important in the latter case.

- *PHA production in bioreactor*

PHA production at the bioreactor scale was then carried-out with a working volume of 2L. As shown in Table 3, and in accordance with the growth curves displayed in Fig.1, the highest cell density was obtained with LPW as growing medium. However, the latter contained the lowest amount of PHA. This result confirms those obtained in shake flasks experiments.

The characteristics of the PHA produced by *Halomonas* i4786 in the different production conditions are listed in Table 4 and Table 5. As it can be shown, despite rather similar molar masses comprised between 5.10^5 and 7.10^5 g.mol^{-1}, the weight average molecular weight and the number average molecular weight are higher for PHA produced on LPW than for PHA produced from FPW. The difference is more pronounced for values (644 500 g.mol^{-1} and 518 000 g.mol^{-1} for LPW and FPW respectively) than for values (677 500 g.mol^{-1} and 588 000 g.mol^{-1} for LPW and FPW respectively) leading to a less uniform distribution for FPW samples compared to LPW ones which have a rather narrow distribution (1.14 and 1.05 for FPW and LPW respectively). This can also be noticed on SEC chromatograms of each PHA represented on Fig. 3. Compared to LPW (dashed line), the FPW sample (solid line) gives a narrow peak shifted to higher elution volumes, which correspond to a less uniform distribution and lower molar masses, respectively.

DSC experiments were also performed to characterize the PHA thermo-induced transitions which allow to investigate the physical PHA behaviours in relation to their structural features. The

corresponding parameters are displayed in Table 5. The melting temperatures (T_m), the glass transition temperatures (T_g) and the melting enthalpy (or crystallinity) of the two produced PHA are relatively dependant to the nature of the culture medium. All these recovered parameters indicate that the thermal behaviours of these PHA differ which could arise either from distinct molar masses distribution, *i.e*. chain length distribution as indicated by the SEC characterization and/or from distinct levels of chain ramifications. The thermal characteristics of PHA copolymers strongly depend on the type, content and distribution of comonomer units comprising the polymer chains, as well as the average molecular weight and molecular weight distribution. The glass transition is influenced by the size of the side group and generally, the observed relationship suggests that an increase in the alkyl chain size of the side group causes steric hindrance and results in a decrease in T_g, mainly due to an increase in the free volume [21]. The higher of alkyl chain length, the lower the T_g of the PHA. The T_g values in Table 5 clearly shows the pronounced depression, indicating the enhanced local segmented mobility of polymer chains. The crystallinity of PHA copolymers is also greatly affected by the type of comonomer as previously shown by Noda et al. [22]. However, GC analyses were performed on LPW and FPW PHA and both chromatograms were similar: each PHA is mainly composed of polyhydroxybutyrate (PHB). Then, the polymer composition cannot be responsible for the differences observed for T_g, T_m and crystallinity. Nevertheless, the significant differences in the dispersity between that of PHA produced using LPW ($Đ$ = 1.05) and that of PHA obtained from FPW ($Đ$ = 1.14) could explain the higher crystallinity for the second one. Indeed, this suggests an enhanced local mobility of the polymer chains in the presence of smaller chains, inducing an increase in the crystallinity degree. The small difference in melting temperatures could be attributed to the difference of the crystallinity degree which is significantly higher for PHA produced from fruit process water (T_m = 172.4°C for 68.3% of crystallinity), compared to PHA obtained using LPW (T_m = 166.9°C for 46.9% of crystallinity).

- **Discussion**

World production of plastic materials was in excess of 300 million tons in 2013. Traditional plastics (petrol-polymers) are produced using mineral oils (fossil-based) whose extraction and production are environmentally damaging. Furthermore, once produced, petrol-polymers are persistent in the environment. For example, the average petro-polymer shopping bag lasts between 100 and 400 years before biodegrading. However, bio-based and biodegradable polymer alternatives exist.

Biodegradable polymers are greatly needed by industry to reduce environmental impact of the very large world production of plastic materials. Bio-sourced, recyclable biopolymers constitute a promising alternative providing their biosynthesis at an industrial scale is cost effective. Cost-effectiveness and economic scalability is necessary to ensure the use of bio-sourced recyclable

polymers will not be restricted to high value added products. To realize the full environmental benefit bio-sourced recyclable polymers must become mainstream, replacing petro-polymers on an industrial scale.

Hence, this study aimed at demonstrating the feasibility of producing, firstly at a laboratory scale, PHA polymers from culture media which are enriched with agro-industries' by-products. Such an approach aims at both significantly reduce the polymer's production cost per ton and the carbon footprint of agro-industries. The comparative study performed here demonstrates that agro-industries' by-products efficiently offer an alternative to an expensive conventional culture medium, the marine broth, for PHA production, especially by replacing glucose by agro-industry effluents. Regarding firstly the biomass production formed after 12 hours of culture, it varies more or less within a log as compared to the marine broth reference medium. Besides it is observed that the lag time, related to the time necessary for the cell's metabolic adaptation, is only increased by two hours. This is of weak consequence in an industrial context since the bacterial cells are usually grown on a continuous batch mode. Hence, it can be concluded that sufficient biomass can be produced from media based on agro-industries' by-products. Special attention was paid to the culture second stage since it is critical for initiating PHA biosynthesis, the experimental conditions for starving cells being not straightforward to determine. The results at hand show that such alternative media yield PHA relative (to cell dry mass) contents above 50% and reach up nearly 80% for the FPW. This finding may arise either from the larger sugar amounts in this medium as compared to the leguminous one, and/or from the fact that fructose is more readily metabolized in the growing phase and further incorporated in PHA units than glucose during the starving stage. Indeed, 50% of sugars are saccharose in the leguminous effluent and fructose in fruit ones.

On a methodological point of view, we also demonstrate that the Nile Red emission spectrum incorporated in PHA granules is correlated to the global amount of the PHA content [20]. This technique was previously designed in our laboratory but the strict correlation with the fluorescence intensity at $543_{exc}/592_{em}$ was not yet demonstrated. This value is easily determined and can thus provide an effective tool for monitoring, in real time, the actual PHA production allowing thereby supplying valuable information for the decision-making support of the bio-fermentation process.

Preliminary experiments were achieved to investigate the physicochemical properties of the PHA produced in these challenging culture media. One observe that PHA synthesized from bacterial cells grown with the leguminous effluent exhibit a rather uniform distribution ($Đ$ = 1.05) in opposite to those synthesized with fruit ones which are less uniform as suggested by the calculated $Đ$ value of 1.14. This structural diversity likely warrant for the observed thermal behaviours of PHA produced from cells grown in these two media. Though glass transitions are observed at near sub-zero temperatures and melting temperatures are close to 170°C for both productions, melting enthalpies

and crystallinities both vary. Considering that both samples are only composed of polyhydroxybutyrate, such distinct thermal properties may arise from distinct chain lengths distributions. Further structural and rheological studies are needed to obtain closer insights on these properties. Here, the relevant point is that these observed behaviours do differ. In the framework of green processes, it is of utmost importance that structural PHA diversity can be achieved by purposely choosing the carbon and nitrogen sources to feed a unique bacterial strain. This limits the need for complex genetic modifications and enzymatic engineering. In other words, from a unique strain, distinct PHA can be synthesized which necessarily will exhibit distinct properties. Hence, it comes at hand to set a specific effluent for cell growth in order to get desired mechanical properties according to the desired plastic material fate, including use and life duration.

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Table 1. PHA produced by pure culture from waste raw materials.

Carbon source	**Organism**	**PHA concentration (g/L)**	**Reference**
Sugar cane	*Bacillus SA*	5.80	[12]
Bagasse	*C.necator*	3.90	[13]
Wheat Bran	*Halomonas boliviensis*	4.00	[14]
Whey	*Methylobacterium sp*	6.12	[15]
Canola oil	*W. Eutropha*	18.27	[16]

Table 2. Agro-industrial substrates composition (in g per 100g).

	LPW	FPW
Protein	**10.9**	**1.6**
Carbohydrates	**64.9**	**94.4**
Glucose	0.3	20.4
Saccharose	28.0	nd
Stachyose	17.2	nd
Fructose	nd	46.8
Ashes	**20.0**	**4**
Calcium	0.1	0.5
Sodium	2.2	0.1
Potassium	6.2	2.1
Humidity	**4.2**	**-**
C/N molar ratio	22.9	371.7

nd: non determined

Table 3. Production of PHA by *Halomonas* i4786 depending on the growing medium type. The PHA content was defined as the percent ratio of PHA concentration to CDW.

Growing medium	CDW ($g.L^{-1}$)	PHA concentration ($g.L^{-1}$)	PHA content (%)
LPW	2.8	1.56	55
FPW	2.3	1.79	78

Table 4. PHA dispersity parameters.

Growing medium	(g/mol)	(g/mol)	*Đ*
LPW	644 500	677 500	1.05
FPW	518 000	588 000	1.14

Table 5. Thermal parameters recovered from DSC experiments. Refer to §2.7 for thermodynamic parameters identification.

	T_g (°C)	T_m (°C)	T_c (°C)	ΔH_m ($J.g^{-1}$)	X_c (%)
LPW	-0.2	166.9	61.0	68.5	46.9
FPW	-5	172.4	63.8	99.7	68.3